DNA ligase I is associated with the 21 S complex of enzymes for DNA synthesis in HeLa cells

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ABSTRACT

Approximately 80% of the DNA ligase activity in HeLa cell extracts is associated with the 21 S enzyme complex that functions in simian virus 40 DNA replication *in vitro* (Malkas *et al.*, *Biochemistry* 29, 6362–6374., 1990). The DNA ligase associated with the 21 S complex was purified extensively and its physical, enzymic and immunological properties characterized as DNA ligase I. The association of DNA ligase I with the 21 S complex of enzymes for DNA synthesis provides evidence for the physiological function of this DNA ligase in DNA replication in human cells.

INTRODUCTION

DNA ligases participate in DNA replication, repair and recombination by catalyzing the formation of phosphodiester bonds between adjacent 5'-phosphoryl and 3'-hydroxyl termini at single strand nicks in duplex DNA molecules (1,2). Three forms of DNA ligase have been demonstrated in mammalian cells. These enzymes, designated DNA ligases I, II and III, can be distinguished on the basis of their catalytic, physical and immunological properties (2–5). DNA ligase I represents the majority of the DNA ligase activity in proliferating cells while most of the ligase activity in resting cells is attributable to DNA ligase II (2,4). The level of DNA ligase III, like that of DNA ligase II, does not increase during regeneration of rat liver (4). Immunolocalization studies showed that DNA ligase I is localized exclusively in the nucleus in non-mitotic cells and its immunofluorescent localization pattern is similar to that observed for DNA polymerase α in mammalian cells (6). DNA ligase I from mammalian cells appears to be directly related to the DNA ligase in yeast cells that was shown to be required for replication, recombination and repair of DNA (7). The gene for human DNA ligase I has been cloned, expressed (8) and the gene assigned to human chromosome 19q13.2–13.3 (9). A mutation in the DNA ligase I gene of a human fibroblast strain (46BR) resulted in retardation of Okazaki fragments during lagging strand DNA synthesis (10). On the basis of this and other evidence it is believed that DNA ligase I participates in DNA replication in

mammalian cells (2). DNA ligase II is considered to have a housekeeping function in mammalian cells such as in DNA repair although its actual physiological function remains unknown (2). A multiprotein 600 kDa complex composed of DNA polymerase ϵ , a 5'→3' exonuclease and DNA ligase III was recently isolated from calf thymus and shown to function *in vitro* in DNA recombination and repair (11).

We showed previously that simian virus 40 (SV40) large T antigen (SV40 T-ag)-dependent, origin specific initiation of SV40 DNA replication *in vitro* in HeLa cell extracts resides with a sedimentable complex of enzymes for DNA synthesis (12,13). DNA ligase activity was reported to be associated with the 21 S enzyme complex (13). The properties or nature of the ligase were not defined in that study, however. Since three distinct DNA ligases exist in mammalian cells, it is of importance to define the actual DNA ligase component of the DNA synthesizing machinery in human cells. In this paper we present evidence that the DNA ligase in HeLa cells that is associated with the 21 S enzyme complex that functions in SV40 T-ag-dependent SV40 DNA replication *in vitro* has the properties of DNA ligase I.

MATERIALS AND METHODS

Materials

Radioactively labeled nucleotides were from Amersham. Unlabeled nucleotides, phenylmethylsulfonyl fluorophosphate (PMSF) and alkaline phosphatase (AP) conjugated goat-antirabbit IgG antibody were purchased from Sigma. Dephosphooligo (dT)₂₅, the 10-mer and 20-mer oligodeoxynucleotides used in the DNA ligase assay were synthesized in the Core DNA Synthesizing Facility at the Worcester Foundation. Sal I and Pvu II restriction endonucleases were from BRL. Mono Q HR5/5 (1 mL) columns and 5' AMP-Sepharose 4B were purchased from Pharmacia LKB Biotechnology Inc. HeLa S₃ cells were grown in suspension culture as previously described (14). Rabbit polyclonal antibody to calf thymus DNA ligase I that cross-reacts with human DNA ligase I (6) was a gift from Dr Tomas Lindahl of the Imperial Cancer Research Fund, U.K. All other reagents that were used have been described in recent publications from this laboratory (12,13,15,16).

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Subcellular fractionation and isolation of the 21 S complex of enzymes for DNA synthesis

The preparation of HeLa cell homogenates, subfractionation of HeLa cell homogenates and partial purification of the 21 S enzyme complex were according to published procedures (12,13). Briefly, the procedure involves poly[ethylene glycol] 8000 (PEG) precipitation of the combined low salt (i.e. 0.15 M KCl) nuclear extract/postmicrosomal supernatant (NE/S-3) from the homogenate and Q-Sepharose chromatography of the resulting supernatant solution (PS). The 21 S complex of enzymes for DNA synthesis and all of the SV40 DNA *in vitro* replication activity in the NE/S-3 fraction of HeLa cell homogenates coelute at KCl concentrations between 0.25–0.4 M during Q-Sepharose chromatography (12,13). The enzymes for DNA synthesis and SV40 DNA replication activity remain associated during velocity gradient centrifugation of the 21 S complex in the presence of 0.15 M or 0.5 M KCl (12,13).

Purification of DNA ligase associated with the 21 S complex

The DNA ligase was dissociated from the 21 S enzyme complex by successive steps of chromatography on columns of native and denatured DNA–cellulose that were coupled in series so that the effluent from the native DNA–cellulose flowed directly onto the denatured DNA–cellulose column. The 0.25–0.4 M KCl eluate from Q-Sepharose containing the 21 S enzyme complex was dialyzed against 50 mM Tris–HCl pH 7.5, 1 mM EDTA–Na₃ (ethylenediaminetetraacetic acid, pH 7.5), 1 mM EGTA–Na₃ (ethylene[bis(oxyethylenenitrilo)]tetraacetic acid, pH 7.5), (buffer-A) containing 0.1 M KCl and loaded onto a column of native DNA–cellulose that was coupled to a column of denatured DNA–cellulose. The DNA–cellulose of both columns was equilibrated with the dialysis buffer. After washing with 10 column volumes of buffer-A containing 0.1 M KCl the columns were uncoupled and each was successively eluted with 8 column volumes of 0.3, 0.5 and 1 M KCl in buffer-A. The DNA ligase activity that is dissociated from the 21 S enzyme complex binds to both native (70%) and denatured (30%) DNA–cellulose and is eluted from each column by 0.3 M KCl (17).

The 0.3 M KCl eluates from the native and denatured DNA–cellulose columns were separately dialyzed against buffer-A containing 50 mM KCl. Fractions containing DNA ligase activity, were pooled and in separate runs were further fractionated by Mono Q FPLC. The dialyzed samples were injected onto a Mono Q HR 5/5 column at a flow rate of 0.5 ml/min. After loading, the column was washed with 5 ml of the loading buffer and eluted with a 20 ml gradient of increasing KCl concentration from 0.05 to 1.0 M. 0.5 ml fractions were collected and the individual fractions dialyzed against buffer-A containing 0.1 M KCl and assayed for DNA ligase activity. In both cases the DNA ligase activity eluted from the Mono Q column at a KCl concentration of approximately 0.2 M.

Several Mono Q FPLC purifications of the DNA ligase in the 0.3 M KCl eluates from native and denatured DNA–cellulose showed that the DNA ligase purified from these DNA–cellulose fractions had identical physical and enzymic properties (17). Thus, the Mono Q FPLC purified DNA ligase activities from 0.3 M KCl eluates of the native and denatured DNA–cellulose columns were routinely pooled, dialyzed against buffer-A containing 50 mM KCl and loaded onto an AMP–Sepharose HR 5/5 column equilibrated with the dialysis buffer. The column was washed with 5 ml of the equilibration buffer at a flow rate of 0.1 ml/min and eluted by a 15 ml gradient of increasing KCl

concentration from 0.05 to 1.0 M at a flow rate of 0.5 ml/min. 0.5 ml fractions were collected, dialyzed against buffer-A containing 0.15 M KCl, 10% glycerol and assayed for DNA ligase activity. DNA ligase activity eluted in a single fraction at approximately 0.6 M KCl concentration. The purified DNA ligase was stored at –80°C.

DNA ligase assays

DNA ligase activity was routinely assayed according to the procedure of Goodchild and Vishwanatha (18). This procedure detects DNA ligase activity by the enzyme joining two decamer oligodeoxynucleotides, one of which is 5'-[³²P] end-labeled, that are hybridized to a complementary dodecamer oligodeoxynucleotide that lacks a 5-terminal phosphate as depicted below

After phenol extraction of the product, ligation of the 5'-[³²P] end-labeled GGGCTGATAA 10-mer oligodeoxynucleotide to the unlabeled (TAGTCTCAAT) oligodeoxynucleotide to form a complementary 20-mer was analyzed by polyacrylamide gel electrophoresis in a 15% cross-linked gel in the presence of 8 M urea (18).

The substrate described above was also used to quantitate the ligase activity by measuring the conversion of 5'-³²P-labeled phosphomonoesters to AP-resistant diesters (4,19). One unit of DNA ligase activity catalyzes the conversion of 1 nmol of terminal phosphate residues to a phosphatase resistant form in 30 min at 35°C.

Since the complementary 20-mer oligonucleotide and the ligated 10-mers in the ligase assay of Goodchild and Vishwanatha (18) lack 5' phosphate termini, blunt-end ligation does not occur. Thus, blunt-end ligation was measured using pBR322 DNA that was digested with Sal I or Pvu II restriction endonucleases. Both of these endonucleases cleave pBR322 DNA at single sites but Sal I produces a product with cohesive ends while Pvu II produces a blunt-end DNA product. The reaction contained 8 µg of pBR322 DNA, 100 units of Sal I or pVU II restriction endonucleases, 80 µl volume of the buffer system supplied by the manufacturer and the digestion was overnight at 35°C. After digestion the DNA product was 5'-[³²P] end-labeled using 10 µg of the oligonucleotide, [³²P]ATP (100 µCi, >5,000 Ci/mmol), 10 µmol ATP and T-4 polynucleotide kinase in the reaction. The 5'-[³²P] end-labeled product was isolated by phenol–chloroform extraction and used as the substrate in the DNA ligase assay. In a similar manner, dephosphooligo(dT)₂₅ 5'-[³²P] end-labeled by the procedure described above and separately annealed to poly(dA) and poly(A). Following phenol–chloroform extraction 5'-[³²P] end-labeled poly(dA) and 5'-[³²P] end-labeled poly(A) were separately annealed with oligo(dT)₂₅. After phenol–chloroform extraction, the annealed products were also used as substrates in the ligase assay. Ligation in all cases was quantitated by conversion of the ligase substrates to AP-resistant forms.

Other assays

The *in vitro* replication of pSVO⁺ plasmid DNA containing an insert of sequences of the replication origin of SV40 DNA was assayed according to the procedure of Wold and Kelly (20) with minor modifications that were described previously (12). One unit of SV40 replication activity is designated as the incorporation of 2 pmol of dNMP into Dpn I resistant SV40 DNA per h under

the standard assay condition. DNA polymerase α activity was assayed according to published procedures (12,13). Protein was determined by the procedure of Bradford (21) using bovine serum albumin as the standard.

Immunoblot analysis

Protein samples were electrophoresed in 8% SDS polyacrylamide gels. Electroblothing of the separated proteins was performed according to the procedure of Towbin and coworkers (22) using nitrocellulose membranes and with minor modifications as previously described (12).

Velocity gradient centrifugation

Sedimentation of the 21 S enzyme complex was performed on preformed 10–35% glycerol gradients prepared in buffer-A containing 0.15 M and formed in polyallomer tubes for the Beckman SW50.1 rotor as described previously (12,13). Sedimentation markers that included porcine thyroglobulin (19 S), horse spleen apoferritin (17 S) and yeast alcohol dehydrogenase (7 S) were run on parallel gradients. After centrifugation at 4°C and 192,000×g for 16 h, 150 μ l fractions were collected by tube puncture from the bottom and assayed for the respective enzyme and SV40 replication activities. Sedimentation values were calculated according to the procedure of Siegel and Monty (23).

RESULTS

Isolation of the 21 S complex of enzymes for DNA synthesis

About 50% of the protein and essentially all of the 21 S enzyme complex and *in vitro* SV40 DNA replication activity in the NE/S-3 fraction are recovered in the PS (12). During Q-Sepharose chromatography of the PS fraction approximately 40% of the protein does not bind to the matrix and appears in the column flow-through/wash fractions. All of the 21 S complex and DNA ligase activity in the PS bind to Q-Sepharose and coelute at a KCl concentration of 0.25 to 0.4 M (13 and Table 1). As shown in Fig. 1, the SV40 DNA replication activity and DNA polymerase α activity cosediment as a 21 S complex during velocity gradient centrifugation of the 0.4 M KCl eluate from Q-Sepharose. The DNA ligase activity resides in fractions within the peak of SV40 DNA replication activity (fractions 8–10) in the gradient. The maximum DNA ligase activity, however, is not exactly coincidental with the peak of SV40 DNA replication

activity associated with the 21 S complex. This is attributable to the association also of the DNA binding, Ku protein and a DNA-dependent protein kinase with the 21 S complex (Cao, Q.-P., Pitt, S., Leszyk, J. and Baril, E., submitted). Both of these proteins bind to the ends and nicks in duplex DNA (reviewed in 25) and inhibit the ligation in the assay of Goodchild and Vishwanatha (18, S.Pitt unpublished data). Importantly, there is no detectable ligase activity in fractions at or near the top of the gradient (data not shown) that should be the position for sedimentation (i.e. 4–6 S) of free DNA ligase I, II or III (3,4,24).

Purification of the DNA ligase from the 21 S enzyme complex

The 21 S enzyme complex is a loosely associated complex and its organization is disrupted during chromatography on coupled columns of native and denatured DNA–cellulose. This procedure was previously used as a step in the purification of the 640 kDa multiprotein DNA polymerase α -primase complex (26) that is exclusively associated with the 21 S enzyme complex (12, 13). Approximately 70% of the ligase activity that is associated with the 21 S complex binds to native DNA–cellulose during chromatography of the enzyme complex and is eluted by 0.3 M KCl (Table 1). The ligase activity that does not bind to native DNA–cellulose, however, does bind to denatured DNA–cellulose and is also eluted by 0.3 M KCl. Topoisomerase I, DNA-dependent ATPase and a small amount of uncharacterized DNA binding proteins that are associated with the enzyme complex coelute with the DNA ligase activities that are eluted from the native and denatured DNA–cellulose columns (13). The 640 kDa multiprotein DNA polymerase α -primase complex (26), RNase H, PCNA and a 5'→3' exonuclease do not bind to the DNA–celluloses, however, and appear in the column flow through from the denatured DNA–cellulose column (13).

Mono Q FPLC

FPLC of the 0.3 M KCl eluates from the native and denatured DNA–cellulose columns on a Mono-Q column separates the DNA ligase from a DNA-dependent ATPase and topoisomerase I activities (Fig. 2A–C). The ligase activity in both cases elutes from the Mono-Q column at a KCl concentration of 0.25 to 0.3 M. The DNA-dependent ATPase and topoisomerase I activities elute at KCl concentrations of 0.35 M and 0.5 M respectively. The topoisomerase that binds to the native and denatured DNA–cellulose columns has the physical and enzymatic

Table 1. Summary of purification of HeLa cell DNA ligase I

Purification step	total protein (mg)	total activity (units) (units/mg)	specific activity	purification (×-fold)
I.PEG Supernatant 2400	5.9	0.019		
II.Q-Sepharose	1536	6.1	0.023	1.2
III.DNA–Cellulose				
Native	8.8	14.7	1.67	88
Denatured	3.8	6.3	1.66	87
IV.Mono Q FPLC of DNA ligase eluted from DNA–cellulose:				
A.Native 0.9	12.1	13.4	707	
B.Denatured 0.7	10.7	10.7	564	
V.AMP–Sepharose affinity FPLC of combined				
IV A & B	0.2	17.6	89	4700

The purification was from 40 g (wet weight) of HeLa cells. The procedures and assays were as described under Materials and Methods

Figure 1. Sedimentation of the SV40 *in vitro* replication, DNA polymerase α and DNA ligase activities associated with the 21S enzyme complex. A 0.2 ml aliquot of the Q-Sepharose eluted fraction containing the peak of SV40 DNA replication activity was layered onto a preformed 10–35% glycerol gradient in buffer-A containing 0.15 M KCl that was prepared in polyallomer tubes for Beckman SW50.1 rotor. The conditions for centrifugation, fractionation of the gradient and assays for SV40 DNA replication, DNA polymerase α and DNA ligase activity were as described under Materials and Methods. **A:** SV40 *in vitro* DNA replication (open circles) and DNA polymerase α (closed circles) activity measured in fractions throughout the gradient. **B:** Polyacrylamide gel electrophoretic analysis of the ligated product from the assay of the DNA ligase activity within the 21 S peak (fractions 8–12). Electrophoresis was performed on a 15% polyacrylamide gel in the presence of 8 M urea according to the procedure of Goodchild and Vishwanatha (18).

properties of topoisomerase I (13, 27). The DNA-dependent ATPase is the same ATPase that was previously reported to be associated with the enzyme complex (16) and has physical and immunological properties of human Ku protein (Cao, Q.-P., Pitt, S., Leszyk, J. and Baril, E., submitted).

5'AMP–Sepharose affinity FPLC

DNA ligases from prokaryotes (1) and eukaryotes form a covalent enzyme–AMP intermediate and this intermediate has been demonstrated for eukaryotic DNA ligases isolated from a variety of sources (2). The reaction requires divalent cations and, as in the case of DNA ligases from T-4 bacteriophage and eukaryotes, ATP as a cofactor. Thus, we successfully utilized AMP-affinity chromatography as the final step in the purification of the DNA ligase that is associated with the 21 S complex of enzymes for DNA synthesis. The 280 nm absorbance profile of the column flow through/wash and the eluted fractions from AMP–Sepharose FPLC are shown in Fig. 3 (upper panel). All of the ligase activity bound to the AMP–Sepharose and eluted as a single peak at a KCl concentration of approximately 0.6 M (Fig. 3 lower panel).

Figure 2. Absorbance 280 nm profile from Mono Q FPLC of the 0.3 M KCl eluted fractions from native and denatured DNA–cellulose chromatography of the 21S enzyme complex. **A:** Mono Q FPLC of the 0.3 M KCl eluted fraction from native DNA–cellulose. Arrows indicate the eluted peaks of DNA ligase, DNA-dependent ATPase and topoisomerase I activity, respectively. The solid line indicates the molar concentration of KCl in eluted fractions throughout the gradient as determined by conductivity analysis. **B:** Mono Q FPLC of the 0.3 M KCl eluted fraction from denatured DNA–cellulose. Arrows indicate the eluted peaks of DNA ligase, DNA-dependent ATPase and topoisomerase I activities, respectively. The solid line indicates the molar concentration of KCl in eluted fractions throughout the gradient as determined by conductivity analysis. **C:** DNA ligase activity in the eluted peak of DNA ligase activity during Mono Q FPLC of the 0.3 M KCl eluted fraction from native (hatched bar) and denatured (solid bar) DNA–cellulose. The procedures used for Mono Q FPLC and assay of DNA ligase, DNA-dependent ATPase and topoisomerase I activities were as described under Materials and Methods. In each case, one-half of the 0.3 M KCl eluted fraction from the DNA–cellulose column was subjected to Mono Q FPLC in separate runs.

Figure 3. AMP-Sepharose affinity FPLC purification of the DNA ligase associated with the 21 S enzyme complex. Upper Panel: Absorbance 280 nm profile of the material in the column flow-through/wash (fractions 0–7) and the eluted fractions (8–20) from AMP-Sepharose of the pooled DNA ligase from Mono Q FPLC of the DNA ligase in the 0.3 M KCl eluates of the native and denatured DNA-cellulose columns respectively. The diagonal line indicates the molar concentration of KCl in eluted fractions throughout the gradient as determined by conductivity analysis. The profile represents one-fourth of the fraction containing DNA ligase from Mono Q FPLC that was fractionated in separate runs of AMP-Sepharose affinity FPLC. Lower Panel: Polyacrylamide gel electrophoretic analysis of the product from the assay of DNA ligase activity in the peak that is eluted from AMP-Sepharose. Aliquots of fractions 9–20 (upper panel) eluted from AMP-Sepharose were assayed for DNA ligase activity and the ligated product separated on a 15% polyacrylamide gel in the presence of 8 M urea according to the procedure of Vishwanatha and Goodchild (18). The arrow indicates the position of the peak of 280 nm absorbance (upper panel) and the peak of DNA ligase activity (lower panel) that eluted at a KCL concentration of 0.6 M. The procedures for AMP-Sepharose affinity FPLC and assay of DNA ligase activity were as described under Materials and Methods.

Table 1 summarizes the data for the purification of the DNA ligase that is associated with the 21 S complex of enzymes for DNA synthesis from HeLa cells. The ligase activity in the PS has been purified about 4700-fold through the AMP-Sepharose affinity FPLC stage of the purification with a recovery of about 40% of the activity. We were unable to quantitate reliably the ligase activity in fractions prior to the PEG precipitation step in the purification due to the presence of nucleases and other interfering activities in these crude fractions. Additional interfering activities such as DNA binding proteins, DNases etc.

Table 2. Substrates for purified HeLa cell DNA ligase I

Substrate	Alkaline phosphatase resistant (cpm)
5'-TAGTCTCAAT[³² P]GGGCTGATAA 3'-ATCAGAGTTA-----CCCGACTATT	141,220
pBR322 DNA linearized with Pvu II endonuclease (blunt-ends)	66,570
pBR322 DNA linearized with SalI endonuclease (cohesive ends)	51,430
oligo(dT).poly(rA)	2,815
oligo(dT).poly(dA)	115,360

The assays contained 0.05 units of the purified DNA ligase (fraction V) and the designated substrate. Procedures for the assay and measurement of alkaline phosphatase resistant [³²P] labeled product were as described under Materials and Methods and (4). In the absence of the ligation reaction only 5% to 7% of the [³²P]-labeled substrates remained resistant to digestion by alkaline phosphatase. The procedures for digestion of pBR322 DNA by restriction endonucleases Sal I and Pvu II and purification of the linearized product by agarose gel electrophoresis were as described under Materials and Methods.

present in the Q-Sepharose eluted fraction are separated from the DNA ligase activity during chromatography on DNA-cellulose. This results in about a 300% increase in the DNA ligase activity recovered at this step in the purification (Table 2.). Since more than 80% of the ligase activity in the PS is associated with the 21 S enzyme complex (13) and all of the 21 S complex is recovered in PS while more than 50% of the protein in the crude NE/S-3 fraction is precipitated by PEG (12), it is most likely that the purification is actually significantly greater than 4700-fold.

Characterization of the properties of the purified DNA ligase

Catalytic properties. The DNA ligase purified from the 21 S enzyme complex has an absolute requirement for ATP as a cofactor in the ligation. The K_m of the purified enzyme for ATP is 2 μ M. The DNA ligase efficiently ligated the two 10-mer oligodeoxynucleotides into a 20-mer oligodeoxynucleotide for the substrate that was routinely used to monitor ligase activity during the purification (Table 2.). One distinguishing characteristic of the catalytic properties of DNA ligase I, from those of DNA ligases II and III, is its ability to ligate blunt-end DNA substrates containing cohesive or non-cohesive ends (2,4,5). Another is that DNA ligase I will ligate poly(dA). oligo(dT) but will not ligate poly(A). oligo(dT) (2,4,5). As shown in Table 2., the DNA ligase purified from the 21 S enzyme complex is able to ligate blunt-end DNA substrates, poly(dA). oligo(dT) but does not ligate poly(A). oligo(dT). Thus, the purified ligase has catalytic properties that are characteristic of DNA ligase I.

The enzyme is DNA ligase I. Purified DNA ligase I is reported to have a molecular mass of 125 kDa (2,3,6). DNA ligase II has a molecular mass of about 72 kDa (2) and DNA ligase III is a 100 kDa protein that is associated with a 46 kDa polypeptide (2,3,5). The results from SDS polyacrylamide gel electrophoresis of fractions during the course of purification of the DNA ligase are shown in Fig. 4A. Polyacrylamide gel electrophoresis under denaturing conditions of the eluted DNA ligase from 5' AMP-Sepharose affinity FPLC showed a single band of 125 kDa by silver staining (lane 5 in Fig. 4A). Partial tryptic digestion of the pre-formed DNA ligase-AMP adduct formed from reaction of the purified, 125 kDa DNA ligase with [α -³²P]-ATP resulted in its conversion to a catalytic active ~85 kDa

Figure 4. SDS polyacrylamide gel electrophoresis and immunoblot analysis of the purified DNA ligase. **A:** Aliquots of fractions at different steps in the purification of the DNA ligase were analyzed by electrophoresis in a 7.5% SDS polyacrylamide gel and the separated proteins visualized by silver staining as described under Materials and Methods. Lane 1: 25 μ g of PS fraction; Lane 2, 20 μ g of Q-Sepharose eluted DNA ligase; Lane 3, 10 μ g of the combined 0.3 M KCl eluted DNA ligase fractions from native and denatured DNA-cellulose; Lane 4, 2 μ g of DNA ligase eluted from Mono Q FPLC; Lane 5, 0.1 μ g of DNA ligase eluted from 5'AMP-Sepharose affinity FPLC. The molecular mass markers were: myosin, 200-kDa; b-galactosidase, 116-kDa; phosphorylase b, 97-kDa; bovine serum albumin, 66-kDa; ovalbumin, 42-kDa; carbonic anhydrase, 31-kDa and soy bean trypsin inhibitor, 21-kDa. **B:** Immunoblot analysis of the purified DNA ligase. An aliquot (0.1 μ g) of the DNA ligase from the 5'AMP-Sepharose affinity FPLC stage of the purification was electrophoresed under denaturing conditions on 7.5% SDS polyacrylamide gel. Following electrophoresis the separated proteins were electro-transferred to a nitrocellulose membrane and immunoblotted according to the procedure described under Materials and Methods. After blocking and washing in TBS the nitrocellulose membrane was incubated at room temperature for 1 h with a 1:100 dilution of rabbit anti-calf thymus DNA ligase I antibody in TBS. This was followed by a 1 h incubation at room temperature with a 1:1000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG in TBS. Color was developed by incubating in a diethanolamine buffer, pH 9.8; containing 4 mM $MgCl_2$, 0.005% 5-bromo-4-chloro-3-indolyl phosphate and 0.01% nitroblue tetrazolium. The molecular mass of the 125-kDa band in the immunoblot was calculated from its migration relative to the migration of the molecular mass markers indicated in Fig. 4A.

polypeptide (17). This is in agreement with the structure that was demonstrated for the purified 125 kDa DNA ligase I from calf thymus (24).

Immunoblot analysis of the purified DNA ligase with polyclonal antibody to calf thymus DNA ligase I that cross-reacts with human DNA ligase I (6,26) confirmed that the 125 kDa band is immunologically DNA ligase I (Fig. 4B).

DISCUSSION

In previous reports we showed that essentially all of the SV40 *in vitro* DNA replication activity in HeLa cell extracts resides with a sedimentable 21 S complex that contains requisite enzymes and nonenzymic proteins for DNA synthesis (12,13). The initiation of SV40 DNA replication by the isolated 21 S enzyme complex is completely dependent on SV40 T-ag and a DNA template containing SV40 core ori DNA sequences. The product synthesized by the enzyme complex is full length, covalently closed, supercoiled (Form I), circular duplex DNA. The synthesis of covalently closed, circular, duplex SV40 DNA molecules suggests that the required components for efficient leading strand and lagging strand synthesis at the replication fork are associated with the 21 S enzyme complex.

This paper reports on the purification and characterization of the properties of the DNA ligase that is associated with the 21 S complex of enzymes for DNA synthesis from HeLa cells. The physical, enzymatic and immunological properties of the purified DNA ligase are very similar to those of calf thymus DNA ligase I that was recently purified to apparent homogeneity (6) and to human DNA ligase I that was partially purified from a human lymphoblastoid cell line (24). Analysis throughout the course of its purification indicated that all of the DNA ligase associated with the 21 S enzyme complex is DNA ligase I.

It is of interest that essentially all of the DNA ligase I protein from calf thymus tissue (28) and bovine cells in culture (6) is extracted in buffers containing 0.1 M NaCl. It was shown previously that essentially all of the 21 S complex of enzymes for DNA synthesis and the SV40 DNA *in vitro* replication activity are extracted from HeLa cells by 0.1 M KCl (12). The intracellular immunolocalization of DNA ligase I in human cells (6,13) was very similar to that observed for DNA polymerase α (29,30) an enzyme that was shown to be essential for the initiation of SV40 DNA replication *in vitro* (31,32). The similar extractions requirements and immunolocalization pattern of these enzymes, that are presumably involved in similar physiological processes, is of obvious interest in regard to the actual nuclear organization of the DNA replication apparatus in mammalian cells.

There is considerable evidence that implicates DNA ligase I in DNA replication in mammalian cells (2). Although it has not yet been demonstrated, DNA ligase I presumably functions in the ligation of replication intermediates (Okazaki fragments) into high molecular weight DNA during lagging strand synthesis at the replication fork as has been shown for the DNA ligase from *Escherichia coli* (33). A mutation in the gene for human DNA ligase I results in a retardation of lagging-strand DNA synthesis (10). In *in vitro* reconstitution studies with HeLa (34) and mouse cell (35) extracts, a cell fraction containing undefined DNA ligase activity was reported to be essential in addition to RNase H, a 5' $\rightarrow$ 3' exonuclease and DNA polymerase α -primase for lagging-strand DNA synthesis. Also, purified calf thymus DNA ligase I in conjunction with calf thymus DNA polymerases α , δ or ϵ

and a 5'→3' exonuclease were reported to complete lagging strand DNA replication *in vitro*, using a synthetic DNA substrate as a model replication fork (36). The exclusive association in HeLa cells of DNA ligase I with a 21 S complex of enzymes for DNA synthesis that functions in the replication of full-length SV40 DNA *in vitro* provides additional evidence for the participation of DNA ligase I in DNA replication.

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